FORM PTO-1390 (Modified) (REV 11-98) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR
07038.0004U1	10/070574

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/08662	5 SEPTEMBER 2000	10 SEPTEMBER 1999

TITLE OF INVENTION

REGULATORY SEQUENCES AND EXPRESSION CASSETTES FOR YEASTS

APPLICANT(S) FOR DO/EO/US

Becher et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ A copy of the International Search Report (PCT/ISA/210).
8. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ Certificate of Mailing by Express Mail
20. ☒ Other items or information:

Diskette containing the Sequence Listing for this application in computer readable form (CRF) and a paper copy of the Sequence Listing in compliance with 37 C.F.R. §§ 1.821-1.825. Applicants hereby certify that the information in both the computer readable form and the paper copy of the Sequence Listing enclosed herewith is the same and includes no new matter.

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U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/070574	PCT/EP00/08662	07038.0004U1

21. The following fees are submitted:.

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

		CALCULATIONS PTO USE ONLY
☐	Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO and International Search Report not prepared by the EPO or JPO	$1,000.00
☒	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but Internation Search Report prepared by the EPO or JPO	$860.00
☐	International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	$710.00
☐	International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)	$690.00
☐	International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)	$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT = $890.00

Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)). $130.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	43 - 20 =	23	x $18.00	$414.00
Independent claims	11 - 3 =	8	x $84.00	$672.00
Multiple Dependent Claims **(check if applicable)**.			☒	$280.00

TOTAL OF ABOVE CALCULATIONS = $2,386.00

Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) **(check if applicable).** ☐ $0.00

SUBTOTAL = $2,386.00

Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). + $0.00

TOTAL NATIONAL FEE = $2,386.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐ $0.00

TOTAL FEES ENCLOSED = $2,386.00

Amount to be: refunded	$
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☒ Credit Card Payment Form PTO-2038 authorizing payment - $2,386.00

☐ Please charge my Deposit Account No. in the amount of to cover the above fees.
A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. 14-0629 A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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REGULATORY SEQUENCES AND EXPRESSION CASSETTES FOR YEASTS

The invention concerns a DNA sequence which is active as a promoter in yeast cells, an expression and optionally secretion system containing the DNA sequence, plasmids containing that system, host cells that have been transformed with the DNA, and methods of producing proteins and polypeptides.

The production of peptides and proteins with gene-technology methods has become the usual practice in the meantime and many different systems are available for that purpose. Frequently, use is made of bacteria systems which however suffer from disadvantages, in particular in regard to producing medicaments or vaccines, for example that they produce pyrogenic substances which have to be removed prior to use, or they cannot glycosylate the polypeptides. Therefore, a series of other systems for the expression of polypeptides in eukaryotic cells have also been developed. An organism which is wide-spread for that purpose is the yeast Saccharomyces cerevisiae, the genome of which is known in the meantime and can be obtained for the vectors and expression systems. However the use of that micro-organism also involves disadvantages. Thus Saccharomyces is for example temperature-sensitive, which requires expensive pieces of equipment for temperature control in culture.

A genus which is considered for bio-technological methods by virtue of advantageous properties is the yeast *Kluyveromyces*. The species *K. lactis* and *K. marxianus* are classified as GRAS (Generally Recognised As Save) and can therefore be used with the same security as Saccharomyces. In addition K. marxianus can use a large number of carbon sources and energy sources for growth and is not very temperature-sensitive. *Kluyveromyces marxianus* can grow at temperatures of up to 45°C and can therefore be more easily cultured, in contrast to the temperature-sensitive *Saccharomyces* strains. The cells of rapidly growing *K. marxianus* strains can divide under optimum conditions every 35 minutes. However it was

hitherto not possible to make optimum use of those good properties as reliable, strong-expression variable promoters are lacking for that type of yeast and there are scarcely any expression systems available, with which proteins can be produced with a high level of effectiveness.

Now, the object of the present invention was to provide a promoter which is suitable for expression in yeasts, in particular yeast cells of the genus *Kluyveromyces*, as well as expression systems which can be used variably for expression.

That object is attained in that in accordance with the invention a DNA sequence which includes the nucleotide sequence in accordance with SEQ ID No 1

GAGGCCTGTC CGATTATTAA ACTTGCGGCA CCCGAGTTTG TGACCTTCGA
CGACATGTTT TATTTCCACA CCGTAGCTAC ACTTTCTATG TAGTAAGTAG
GTAGTATGGA TGGTAGCTAG TAGAAACTAA ACGAAACGAA ATAAATGTGA
AATGTTAGAC GTAAAGGGGA GGGGAAGGGA AGGGGGCGGC GGAGAGACAT
GCCAGCCAT GCCATTTCAT GGCATGGCAT GTCAAGGGAT ACTGCATGCA
TGCATGCATA CTTTACCAAT AGCAAAGTAA ATTGCTTTCT TCCCCCATTT
GAAACTATTC CACCTCAATC CATCTTTTCT ATAATGGGTA TCACCGATCT
CATGTGTTCT AATAATGCTG CAGGCAACAA CAAATCTTAA AGGCAACTTG
GAATGTAATT TGGTTAATGA TAGATATCAA ACAGCAATGG TGGGCTCCAA
CCGCATGGAT ATGCTCACCT TATTATCCGG AATTGTTGTT CCGCAGGAAA
AAAAAAAAAC CTCGAACCAG ATATTAATTA TCCTATCATT ACTGCGTACA
AAACCCGGGA ACGGTTAACC TGCAGCAGCC GTTTTGCTTA CAGTTCTCAT
GCACAATCAG CCAGATTTTG CAATAGTATT AACTTAGAAT TAAGGCAACA
TCTTTGGATA TGCATGTAGA GTAAGTCGTT CGAAACCATT ATTATTATTA
TTATTATTAT TATTATTATT ATTATTATTA TTATTAGTAT TATTGAAATT
GTTATTGTTC TTAGTTTCAC TACTATTATT ATTCATATTC ATGTTATTGA
CATCGCCGAA CGACCAGCCT CCATACCGAT TAGACAGGAT CTCAAACGTG
GGCTCCAGAG CTCACACATT ATGCTAAATA ACTATCTACT GTAACAGCTA
CAGAAAAAAA ACTATAAAAG AGCGAGGAT AAACCACTCT CTTGTGAATC
AGGATCAGTA GGTAACTCAT AAACCTTCTT CTTTTCTCTC AAAATATCAA
ATAACAGTAG TATCAACAAC GATATCGAAT AATACTAACT ACTACAACAG
TAGGAACAGT AACGACAACG ACAACGATAG TAACGACAAT AACGACACCA

ACAAACAACA GGAACACAGA TTAAGCTCAG AAACAAAAAA AAAAAA

is provided.

The nucleotide sequence according to the invention includes a sequence which is active as a promoter and furnishes an expression system which is highly variable and suitable for *Kluyveromyces*, but can also be used for other types of yeast, in particular Saccharomyces *cerevisiae*.

The DNA sequence according to the invention, in accordance with SEQ ID No 1 is a nucleic acid sequence which contains regulatory regions of a gene which codes for the enzyme endopolygalacturonase. The enzyme endopolygalacturonase breaks down pectin insofar as it cleaves 1,4-α-D-galactosituronic bonds between two non-methylated galacturonic acid esters. That enzyme occurs inter alia in yeast strains of the type *Kluyveromyces marxianus*. The sequence of the endopolygalacturonase gene from *Kluyveromyces marxianus* var. marxianus was published in Yeast 15, 311-322 (1999). The promoter region which can be derived from the stated sequence however did not reliably result in the expression of various proteins.

It has now been found that a sequence which includes a part of the nucleotides of SEQ ID No 1, preferably at least the nucleotides 1 to 1134 and in particular the entire sequence permits the expression of proteins in a highly advantageous and reproducible fashion. The claimed sequence in accordance with SEQ ID No 1 has a plurality of regulatory components so that it can perform its function as a promoter under the most widely varying conditions. The promoter according to the invention can be induced by the addition of pectin to the culture medium. That is advantageous as pectin is a readily available substance and thus an advantageous induction agent is available for the system according to the invention.

The described nucleic acid sequences with a regulatory activity include such sequences which have occurred by virtue of modification, substitution, deletion or insertion or combinations thereof, which involve the same or better regulatory activity than the promoter, the terminator or the signal sequence. For that purpose it is also possible to include the

sequence in accordance with SEQ ID No 1 with further regulatory upstream sequences with activator and/or repressor functions.

In accordance with the invention moreover such sequences are also considered, which with the claimed nucleotides or sequences have a homology of at least 80%, preferably at least 90% and in particular 95%, as long as they also have comparable activity.

Preferably the sequence in accordance with SEQ ID No 1 is afforded in the form of an expression system or an expression cassette for the expression of proteins and peptides. The expression cassette according to the invention includes in its simplest form a regulatory sequence as is shown in SEQ ID No 1 or a part thereof which is active as a promoter, an insertion cloning site into which the polynucleotide can be cloned for the protein to be expressed, and the nucleotide sequence in accordance with SEQ ID No 2

GCGTCTCT TTTTATTTTT TTTTTTTTTT TTATTAACGT GAAGAAGATA
AGGGAAGTCT TCAATGCGGT TCTGAATGGT TGATCCATTT CGATACCTCG
GGGACTTCCT TTGAATATAT TCTGAGAGTA TGACAGTTGG TTTTCTTTCT
TTCTTTCTAT TGTTTTTGTT TTTATGGAAA TATAGCTTTG ATGATTTAGG
ATATTTTTTG TAGTGAACCA ATACATGCTT GATTAATATA CGTACGAGGT
GGGCATTCTA CTCTCATTAT TGGTGTTTTA TTGGAGGAA AAATTAAATC
TAGGAGTATC GTTTAGAGCG CGAACGTAAT ATCCATGTTC TTCTCTTTGA
AGAGGTCCCA CCATTGCTTC CCAGATGCC AGCATTCTTC CATGATATTT
TGCGCTTGTT TTGCACTGGT GACACCCTTT CGAACCAAAG ATGTCAAGTG
CTGCTGATAC AACAACCTGT ATTCATACAA TTCTGGATCC ATCAGCTCAC
AATCCACAGC TGAAGATACA GAAAATGATA CATGTCTCTG CAG

which codes the terminator sequence region of the endopolygalacturonase gene from *Kluyveromyces marxianus*. That expression cassette can be used in many different ways. The insertion cloning site is a cutting location at which the sequence can be cut open and the polynucleotide for the desired protein or peptide can be ligated in. In that simplest form after induction upon expression the protein is intracellularly produced and is not removed from the cell. It can then be obtained in per se known manner after opening the cell. This embodiment is suitable both for small peptides and

proteins which are unstable outside the cell and also for proteins which are generally intracellularly located.

In a further embodiment which is suitable in particular for proteins which are to be removed from the cell, use is made of an expression and secretion system according to the invention. That system includes in operative association the nucleic acid sequence in accordance with SEQ ID No 1 or a part thereof which is active as a promoter, the sequence in accordance with SEQ ID No 2 as a terminator and, between those two sequences, the signal sequence in accordance with SEQ ID No 3

ATGT TATTCAGCAA CACCTTATTG ATCGCAGCAG CTAGTGCATT
ATTAGCTGAA GCTTCTCCAT TGGAAAAGAG A

for removal of the protein. In this embodiment culture is effected in per se known manner, wherein either in a continuous method the protein is continuously discharged into the medium and can be continuously obtained from the fermentation liquor or in a discontinuous method the cells are cultivated, harvested and then the protein can be obtained from the liquor.

The expression cassette according to the invention is suitable both for the expression of suitable autonomously replicating plasmids and also for incorporation in yeast chromosomes by way of integrative vectors.

A further subject of the invention is the plasmids pEPG1-1, pEPG1-2 and pEPGsec which are described in greater detail with reference to Figures 1 to 3 and which contain the expression systems according to the invention. Those plasmids are recombinant bacterial plasmids and can be used in the present form for amplification of the expression cassettes. The plasmids are contained in the micro-organisms DSM 12919, DSM 12920, DSM 12921 or DSM 12922* and are deposited therewith [*Deposited at DSMZ, Mascheroder Weg 1b, 38124 Brunswick].

It is however preferred for the plasmids, after the desired polynucleotide has been ligated in for the expression of a peptide or protein, to be amplified in E.Coli, then to obtain the plasmids, to cut out the expression cassette with suitable restriction endonucleases for which cut locations are provided at the edges of the expression cassette, and to ligate the expression cassette into a yeast vector. The vectors usually contain

selection markers in order to be able to select successfully transformed cells in per se known manner.

The plasmids can possibly be multiplied in *E. coli* and then put into *Kluyveromyces marxianus* or another *Kluyveromyces* strain or also another yeast strain. The transformation system used can be for example known plasmids based on the *Kluyveromyces drosophilarum* plasmid pKD1. Derivatives of that plasmid are suitable for use in *Kluyveromyces marxianus* and when using the expression system according to the invention result in effective expression and secretion of foreign proteins in the corresponding host.

In another embodiment it is possible for the expression cassette including the polynucleotide to be expressed to be cut out of the plasmids according to the invention, when prepared as above, and brought into contact as linear or circularised DNA-strand as an integration cassette directly with yeast cells in order to be absorbed thereby. By virtue of the homology with the endopolygalacturonase gene, then in a part of the treated cells, the DNA is received into the corresponding chromosome by exchange with the endogalacturonase gene. The selection of successfully transfected yeast cells is effected in this embodiment by way of the differing utilisation of pectin.

The expression cassette according to the invention is stably incorporated into chromosomes and, if the cells are cultured under optimum conditions, results in a good yield of the desired protein. The copy number of the system can be set in dependence on the nature of the peptide or protein to be expressed. As the endopolygalacturonase gene is only singly present in the chromosome set of the yeast, upon transfection or transformation with the expression system provided in accordance with the invention a copy of the expression vector is also present only for each successfully transformed cell. If a higher number of copies is desired, then sequences of a gene which is present in the chromosome set in a larger number of copies, for example for rDNA, is ligated in per se known manner to the ends of the expression cassette in order to cause a higher number of exchange events.

In the latter case a marker is additionally also incorporated in per se known manner into the sequence so that the successfully transformed cells can be selected. Methods and markers which are suitable for same are known to the man skilled in the art and do not need to be discussed in greater detail here.

The system according to the invention is highly variable. Thus for example only the sequence in accordance with SEQ ID No 1 or a part thereof which is active as a promoter can be combined with other nucleic acid sequences which provide further regulatory sequences and with a heterologous nucleotide sequence. The sequence in accordance with SEQ ID No 1 or a part thereof which is active as a promoter can be combined with the sequence of SEQ ID No 2 in order to provide a regulatory system which is homologous in *Kluyveromyces marxianus* and into which the polynucleotide for the protein to be expressed is inserted or it is possible for a system of SEQ ID No 1, SEQ ID No 2 and SEQ ID No 3 to be combined together with a gene to be expressed, which codes a desired protein, in order to produce a product which is to be discharged into the culture. As *Kluyveromyces* cells can be grown with many different C-sources and are not very demanding in respect of further nutrients and moreover are temperature-insensitive, this affords a highly effective system. Reliable expression of the foreign proteins is achieved by the regulatory sequence provided in accordance with the invention.

In accordance with the invention, there is provided a system which makes it possible to use the type of yeast *Kluyveromyces marxianus* which is highly promising as a host by virtue of its remarkable physiological capacities.

The system according to the invention is suitable for the expression of peptides, polypeptides, proteins and hybrid molecules including glycosylated proteins.

Thus in a further embodiment the expression cassette may also include the complete sequence of the endopolygalacturonase enzyme or parts thereof, wherein a sequence for a desired protein is ligated in between the endopolygalacturonase gene and the terminator sequence.

Then, in this embodiment, upon expression, a hybrid is obtained, from which the endopolygalacturonase is separated in per se known manner.

Set out hereinafter are some definitions for terms which are used in the description.

Accordingly, an 'expression vector' is a DNA molecule which can be linear or of a ring shape and which contains a segment which codes a sequence for a protein or peptide which is of interest, which is operatively joined to regulatory sequences. Those regulatory sequences include at least promoter and terminator sequences. The expression vector can additionally contain selectable markers and further regulatory elements and must permit transmission and multiplication in host cells. Replication of the expression vectors can occur autonomously or by integration into the host genome.

The expression 'DNA' or 'polynucleotide' includes polymer forms of deoxyribonucleotides and ribonucleotides of any length and any modification in single- and double-stranded form.

The expression 'operatively joined' means that the individual segments are so arranged that they serve the intended purpose, that is to say they can inhibit transcription and can promote expression from the replication starting point to the termination sequence.

The claimed sequences may have further short sequences which do not interfere with the biological activity of the molecule. In addition the claimed sequences also include allele variants of the sequence, that is to say alternative forms of the gene which have occurred due to mutation.

The term 'protein or peptide' relates to a molecular chain of amino acids with biological activity. The proteins and/or polypeptides can be modified in vivo or in vitro, for example by glycosylation and phosphorylation. The term hybrid molecules is used to denote molecules which include both homologous parts and also heterologous parts, for example such proteins which include a combination of endopolygalacturonase or parts thereof with a foreign protein, or for example nucleotide sequences in which DNA from *Kluyveromyces marxianus* is combined with DNA from other micro-organisms.

The expression cassette according to the invention is suitable inter alia for yeasts of the strains Kluyveromyces and Saccharomyces and is preferably used in yeast strains of the type *Kluyveromyces maxianus var. marxianus*. A particularly preferred strain with particularly advantageous expression properties is *Kluyveromyces maxianus var. marxianus* BKM Y-719 which was described by Siekstele et al 1999 (Yeast 15, 311 - 322 1999)).

A further subject of the invention is a method of producing a recombinant protein which is characterised in that a yeast cell is transformed or transfected with an autonomously replicating plasmid which includes an expression cassette according to the invention and a polynucleotide which codes a foreign protein, the yeast cell is cultured under conditions which are suitable for the expression of the foreign protein, and the protein is obtained.

The subject of the invention is also a method of producing a recombinant protein which is characterised in that an expression cassette according to the invention is brought into a yeast cell where the expression cassette is incorporated into a chromosome, the cell is cultured and then the protein is obtained. Particularly preferably the expression cassette according to the invention is used as a module which permits the construction of episomal or integrative expression vectors which contain the regulatory sequences in accordance with SEQ ID No 1, No 2 and/or No 3.

The expression system according to the invention is suitable for the expression of various heterologous proteins. Particularly preferably the system is used for the expression of HBVS-antigen (hepatitis B virus, surface antigen) and virus protein 1 from polyoma virus. Those proteins are antigen proteins and can be particularly advantageously used as vaccines.

The invention is described by means of the accompanying Figures and the following Examples.

Figures 1 to 4 show plasmids with the expression cassettes according to the invention and Figure 6 shows the primers used in Example 2.

Figure 1 shows the plasmid pEPG1-1. That plasmid includes an expression cassette with the promoter according to the invention in

accordance with SEQ ID No 1 (referred to as EPG1prom), an insertion cloning site which can be cut with BspT1, and the terminator sequence according to the invention in accordance with SEQ ID No 2 (referred to as EPG1term). That expression cassette was ligated into the multicloning site of the plasmid pUC19. The insertion cloning site which can be cut with BspT1 replaces the open reading frame of the endopolygalacturonase gene which was removed.

Figure 2 shows the plasmid pEPG1-2. That plasmid contains the expression cassette according to the invention, which includes a sequence in accordance with SEQ ID No 1 with the nucleotides 572 to 1134 (referred to as EPG1prom) which is active as a promoter, an insertion cloning site which can be cut with BspT1, and a terminator sequence which includes the nucleotides 28 to 541 of SEQ ID No 2 (referred to as EPG1term). The insertion cloning site replaces the open reading frame of the endopolygalacturonase gene inclusive of the sequences -1 to -12 and 1087 to 1115 of that gene.

Figure 3 shows the plasmid pEPGsec. That plasmid contains an expression and secretion system of the invention. The plasmid contains an expression cassette with a promoter in accordance with SEQ ID No 1 (referred to as EPG1prom), a terminator in accordance with SEQ ID No 2 (referred to as EPG1term), a signal sequence in accordance with SEQ ID No 3 (referred to as EOG1lyd) and two cut locations in order to insert the desired nucleic acid sequence for the protein to be expressed. That expression cassette was ligated into the multicloning site of the plasmid pUC19. The open reading frame of the endopolygalacturonase gene was removed from positions 75 to 1084 and replaced by a linker with the cloning sites Eco 1471 and BpU 11021.

Figure 4 shows the plasmid pUC19PG. A 2.198 kb PstI-DNA-restriction fragment from a recombinant lambda GEM™-12-bacteriophage of a genomic gene bank of *Kluyveromyces marxianus* was ligated into the PstI-restriction location of the multicloning site of the plasmid pUC19.

Figure 5 shows the plasmid pUC19-PG1a. A 2.735 kb StuI-PvuII-DNA-restriction fragment from a recombinant lambda GEM™-12-bacteriophage

of a genomic gene bank of *Kluyveromyces marxianus* was exchanged in the plasmid pUC19 for the 321 kb fragment. The bold part of the plasmid corresponds to the DNA fragment from K. marxianus while the thin-printed part represents the proportion of the plasmid pUC19.

Figure 6 shows the primers in accordance with SEQ ID Nos 4 and 5, which are used for the cloning of promoter and signal sequence.

Figure 7 shows the signal sequence in accordance with SEQ ID No 3 and the associated signal peptide (with sequence for pre- and prepropeptide) of the endopolygalacturonase from Kluyveromyces marxianus.

Four micro-organisms which contain the plasmids in accordance with Figure 1, Figure 2, Figure 3 and Figure 4 were deposited at the DSMZ in accordance with the requirements of the Budapest Treaty. *E.coli* pEPG1-1 is deposited under deposit number DSM 12919, *E.coli* pUC19PG is deposited as DSM 12920, *E.coli* pEPGseq deposited under the number DSM 12921 and *E.coli* pEPG1-2 under the deposit number DSM 12922.

Example 1

Manufacture of the basic plasmid for the production of the expression casssettes

A Pst1-DNA-fragment with 2.198 kb, which contains the complete gene of the endopolygalacturonase (EPG1) with the regulatory sequences was inserted into the PstI-cut location of the multicloning site of the bacterial plasmid pUC19. That construct is shown in Figure 4. Using in each case two opposite primers which at the 5'-end bore a recognition site for the restriction enzyme BstT1 and at the 3'-end have homology with the promoter, signal and/or terminator sequence of the EPG1-gene, it was possible by means of PCR passing around the plasmid to produce a DNA fragment which after restriction with BspT1 could be circularised with a ligase. When using different recognition sites for restriction enzymes at the 5'-ends of the PCR primers, suitable linkers had to be ligated in for circularisation ligation or the primers contained additional sequences at the 5'-end, which represented a common recognition site for the restriction enzyme. Depending on the respective choice of primers, defined areas of

the EPG1 region can be deleted from the plasmid by restriction and subsequent circularisation. The recombinant deletion plasmids were amplified in *Escherichia coli* and served as a basis for the expression cassettes. By way of the various recognition sites for restriction enzymes in the multicloning site of the plasmid pUC19 the cassette can then be cut out and cloned into an episomal or integrative vector for a suitable yeast strain. The cassette can also be used directly as a PstI-DNA-fragment for integration into a yeast host strain.

Example 2

Manufacture of a recombinant plasmid with sequence ID No 1, No 2 and No 3

In the bacterial basic plasmid pUC19 the 321 bp PvuII fragment was replaced by a StuI/PvuII DNA fragment of 2.735 kb which contains the complete endopolygalacturonase gene inclusive of the regulatory sequences -1142 to -1 and +1087 to 1595 from *Kluyveromyces marxianus*. The StuI/PvuII fragment was for that purpose isolated from a recombinant lambda GEM™-12-bacteriophage of a genomic gene bank of *Kluyveromyces marxianus*. A map of the plasmid produced: 'pUC19-PG1a' is shown in Figure 5. That plasmid can be used in a similar manner to Example 1 for the production of expression cassettes. As the multicloning site was completely deleted by the cloning strategy with that plasmid and the StuI location in the upstream promoter region of the EPG1 gene was destroyed by ligation with the PvuII location in the pUC19, the PvuII site which has remained downstream in relation to the EPG1 gene and for example the unique NarI site in the lacZ-part of the pUC19 can be used for cutting out the cassette.

By means of the PCR and using the primers shown in Figure 6 (SEQ ID No 4 and No 5) it is possible to generate a DNA fragment which contains the promoter region and the signal sequence in accordance with SEQ ID No 1 and No 3. The degenerated rPEPG primer has in the 5' region a mismatch with respect to the EPG1 sequence in the region 1220 and thereby generates from the EPG1 sequence: CAGTTG a PvuII site (CAGGTG). That point mutation (transition) leads to an amino acid exchange insofar as a cysteine codon is changed from TGT into an arginine codon after CGT. That

exchange occurs however in the proportion of the endopolygalacturonase which is replaced by the reading frame of a foreign gene and therefore has no influence on the expression. The StuI/PvuII-PCR fragment can be inserted as a blunt end into the multicloning site of a corresponding vector. By way of the PvuII site it is possible 'in frame' to add open reading frames of genes whose expression product is to be removed from the cell.

Example 3

Manufacture of polyoma JCV MAJOR COAT PROTEIN VP1:

For the expression of the JCV VP1-gene the expression cassette pEPG1-1 was used. The VP1 gene was amplified by means of PCR using the primers:

JC5 (SEQ ID No 6): 5'TCAAGCTTAAGAATGGCCCCAACAAAAAGA 3'

and

JC3 (SEQ ID No 7): 5'GTAAGCTTAAGATTACAGCATTTTTGTCTG 3'

and cloned as a *Bsp*T1 fragment into the expression cassette of pEPG1-1. The cassette was cleaved with *Pst1* and the DNA fragments were converted at their ends by means of T4 polymerase to blunt ends.

The 'blunt ended' cassette was inserted into the *Sma1* site of the *Kluyveromyces* vector pKDSU. The transcription direction of the *VP1*-gene is in that case opposite to transcription of the *S.cerevisiae URA3* gene.

The recipient (BKM Y-719 *ura*) was transformed with the expression construct and uracil prototrophic clones were isolated. The presence of the expression cassette with the reading frame of the VP1 gene was checked by means of PCR and the above-indicated primers. Selected clones were cultivated in various liquid media. The yeast cells were harvested by centrifuging and broken up in lysis buffer (10mM TRIS-HCl, pH 7.5, 0.01% TRITON x 100, 1mM $CaCl_2$, 100mM NaCl with 1mM PMSF) with micro-glass beads at 0°C. The glass beads were then sedimented by centrifuging (2000 rpm) for 10 minutes. The supernatant material was arranged in a layer on a 20% saccharose pad (in lysis buffer) and centrifuged for 2 hours at 4°C and 35,000 rpm (Beckman L8-75 rotor SW41). The pellet was suspended in lysis buffer and arranged in a layer on a CsCl-gradient (1.24 to 1.38 g/ml CsCl). The centrifuging operation was effected at 4°C, 35,000 rpm for 16

hours in the SW41 rotor. 'Virus like particles' of the JCV VP1 of an average particle size of 45 nm were obtained from the fractions between 1.3 and 1.34 g/ml CsCl.

Example 4

Manufacture of hepatitis B virus surface antigen (HBV s-antigen):

The expression cassettes pEPG1-1 and pEPG1-2 were used for expression of the HBV s-antigen gene of subtype (AYW). The HBS gene was amplified by means of PCR using the primers:

HB5 (SEQ ID No 8): 5'AGCCTTAAGATAATGGAGAACATCACATCAGG 3'

and

HB3 SEQ ID No 9): 5'TGACTTAAGTTAAATGTATACCCAAAG 3'

and cloned as *Bsp*T1 fragment into the expression cassettes. The cassettes were cleaved with *Bam*H1 and the DNA fragments filled at their ends by means of Klenow polymerase to form blunt ends.

The 'blunt ended' expression cassettes were inserted into the *Sma*1 site of the Kluyveromyces vector pKDSU. The transcription direction of the HBS gene in that case is opposite to the transcription of the *S.cerevisiae* URA3 gene. The recipient (BKM Y-719 ura) was transformed with the expression constructs and uracil prototrophic clones were isolated on selective medium. Whole DNA was isolated from selected clones and used for detection of the expression cassette by means of PCR. Positive clones were cultivated in liquid, synthetic and in complete media and used for the production of S-protein particles. Corresponding yeast cultures were harvested by centrifuging and broken up in a PBS buffer with 1mM PMSF with micro-glass beads at 0°C. The glass beads were then sedimented by centrifuging (2000 rpm) for 10 minutes. The yeast membrane fraction was sedimented from the supernatant material by centrifuging for 30 minutes at 14,000 rpm (J20 Rotor Beckman J2-21). HBV s-antigen sedimented with that fraction and was eluated by 0.5% Tween-20 in PBS from the fraction. After renewed centrifuging at 14,000 rpm the supernatant material was obtained and separated off by CsCl-density gradient centrifuging. Highly purified HBV s-antigen particles were isolated from the 1.2 g/ml CsCl fraction.

The yield of isolatable antigen, in cultivation in synthetic media, with the pEPG1-2 cassette, was twice as high as with the pEPG1-1 cassette.

CLAIMS

1. A DNA sequence including the nucleotide sequence of SEQ ID No 1.

2. A yeast expression system containing in operative junction the nucleotide sequence of SEQ ID No 1 or a part thereof which is active as a promoter, an insertion cloning site and the nucleotide sequence of SEQ ID No 2 or a part thereof which is active as a terminator.

3. A yeast expression and secretion system including in operative junction a sequence in accordance with SEQ ID No 1 or a part thereof which is active as a promoter, the nucleotide sequence of SEQ ID No 3, an insertion cloning site and the nucleotide sequence of SEQ ID No 2 or a part thereof which is active as a terminator.

4. Plasmid pEPG1-1 containing a yeast expression cassette according to claim 2 deposited under the deposit number DSM 12919.

5. Plasmid pEPG1-2 containing a yeast expression cassette according to claim 2 deposited under the deposit number DSM 12922.

6. Plasmid pUC19PG deposited under the deposit number 12920.

7. Plasmid pEPGsec containing a yeast expression cassette according to claim 3 deposited under the deposit number DSM 12921.

8. An expression vector containing in operative junction a promoter with the sequence of SEQ ID No 1 or a part thereof which is active as a promoter, a polynucleotide which codes a foreign protein, and a terminator sequence.

9. An expression vector according to claim 8 which in addition also includes a signal sequence between promoter and polynucleotide.

10. An expression vector according to claim 9 characterised in that the signal sequence is a sequence in accordance with SEQ ID No 3.

11. An expression vector according to one of claims 8 to 10 characterised in that the polynucleotide codes an antigen protein or peptide.

12. An expression vector according to claim 11 characterised in that the polynucleotide codes a hepatitis B surface antigen, VP1 from polyoma virus or protein A from Staphylococcus.

13. An expression vector according to one of claims 8 to 12 characterised in that the vector is an integrative or episomal vector.

14. An expression vector according to one of claims 8 to 12 characterised in that the vector is a plasmid replicatable in yeast.

15. A host cell that has been transformed with an expression vector or a plasmid according to one of the preceding claims.

16. A host cell according to claim 14 characterised in that it is a cell of the type Kluyveromyces marxianus.

17. E.coli pEPG1-1 deposited under the deposit number DSM 12919.

18. E.coli pUC19PG deposited under the deposit number DSM 12920.

19. E.coli pEPGseq deposited under the deposit number DSM 12921.

20. E.coli pEPG1-2 deposited under the deposit number DSM 12922.

21. A method of manufacturing a recombinant protein, characterised in that a yeast cell is transfected or transformed with a plasmid which includes the expression cassette according to one of claims 2 and 3 and a polynucleotide which codes a foreign protein, the yeast cell is cultured under conditions which are suitable for the expression of the foreign protein, and the protein is produced.

22. A method of manufacturing a recombinant protein characterised in that an expression cassette according to claim 2 or claim 3 is put into a yeast cell where the expression cassette is incorporated into a chromosome, the cell is cultured and then the protein is obtained.

23. Use of a DNA sequence according to SEQ ID No 1 as a promoter for the expression of foreign proteins in yeast cells.

(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

BERICHTIGTE FASSUNG

(19) Weltorganisation für geistiges Eigentum
Internationales Büro

(43) Internationales Veröffentlichungsdatum
22. März 2001 (22.03.2001)

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(10) Internationale Veröffentlichungsnummer
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(51) Internationale Patentklassifikation[7]: C12N 15/56, 9/24, 15/81

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199 43 383.6 10. September 1999 (10.09.1999) DE

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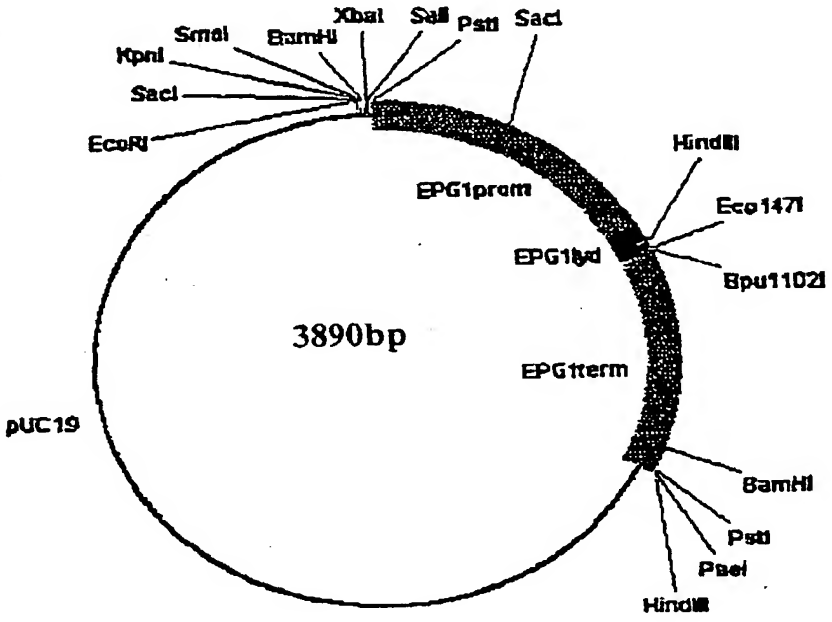
[Fortsetzung auf der nächsten Seite]

(54) Title: REGULATORY SEQUENCES AND EXPRESSION CASSETTES FOR YEASTS, ESPECIALLY FOR KLUYVEROMYCES

(54) Bezeichnung: REGULATORISCHE SEQUENZEN UND EXPRESSIONSKASSETTEN FÜR HEFEN, INSBESONDERE FÜR KLUYVEROMYCES



(57) Abstract: The invention relates to a DNA sequence which acts as a promoter in yeast cells, to an expression and optionally, secretion system containing said DNA sequence, to plasmids containing this system, to host cells that have been transformed with said DNA and to methods for producing proteins and polypeptides.

[Fortsetzung auf der nächsten Seite]

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Expression cassete with BspTI insertion cloning site ligated into the multicloning site of the plasmid pUC19. The open reading frame of the endopolygalacturonase gene has been removed.

pEPG1-1

gaacaaaaaaaaaaaaATG...(ORF EPG)...TAAgcgtctctttttt

1/ 1083

gaacaaaaaaaaaaaaACTTAAGcgtctctttttt

BspTI

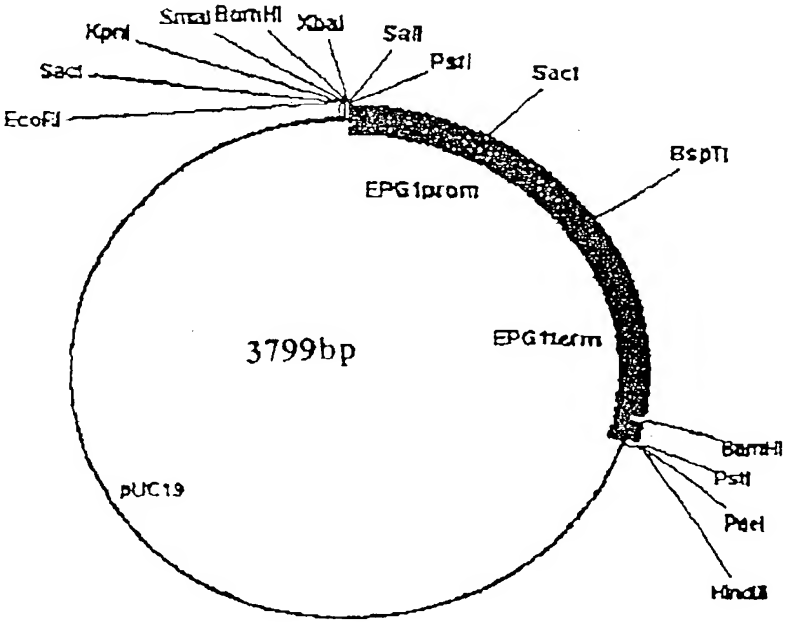

Fig. 1

Expression cassete with BspTI insertion cloning site ligated into the multicloning site of the plasmid pUC19. The open reading frame of the endopolygalacturonase gene including the sequences of -1 to -12 and 1087 to 1115 has been removed and replaced by a BspTI cloning site.

pEPG1-2

-32 cacagattaa gctcagaaaC(aaaaaaaaaa aa ATG...ORF EPG...TAA gc gtctcnnn annnnnn nnnn)Tat taacgtgaag aag

.....cacagattaa gctcagaaaCTTAAG Tat taacgtgaag aag.....

BspTI

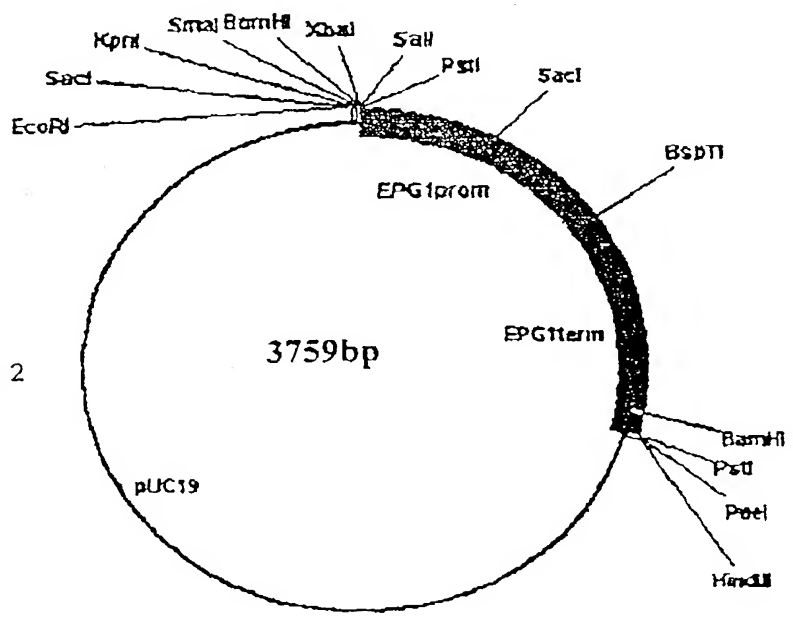

Fig. 2

Expression cassette with secretion signal sequence ligates into the multicloning site of the plasmid pUC19. The open reading frame of the endopolygalacturonase gene was removed from position 75 to 1084 and replaced by a linker with the cloning sites Eco1471 and BpU1021.

pEPGsec1

K R Stop
.....AAG AGA.....(ORF EPG).....TAA GCG TCT.....

K R Stop
.....AAG AGg cct agt gag cct tct gcT AAG CG TCT.....

Eco147I Bpu1102I

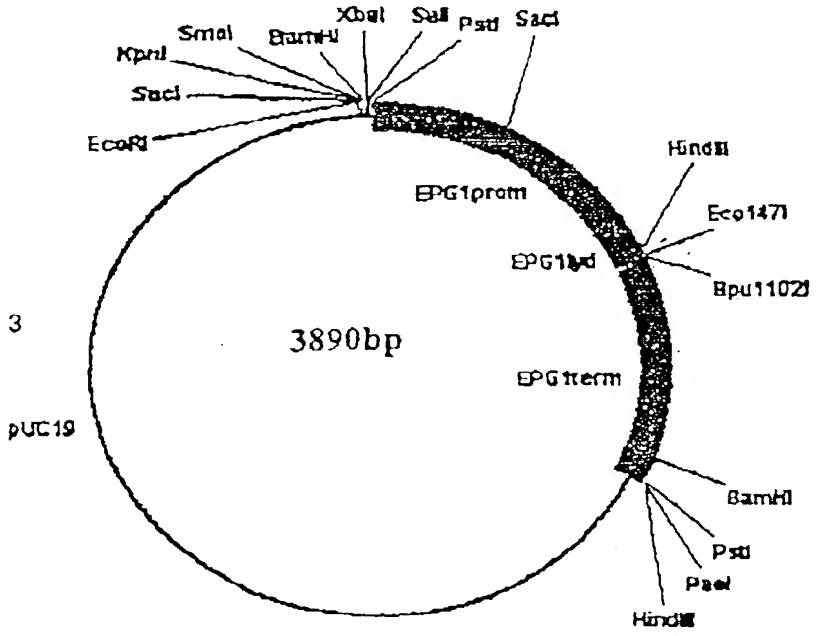

Fig. 3

Plasmid pUC19PG
A 2.198 kb PstI DNS restriction fragment from a recombinant LambdaGEM™-12 of the *Kluyveromyces marxianus* gene bank was ligated into the PstI restriction site of the multicloning site of the plasmid pUC19.

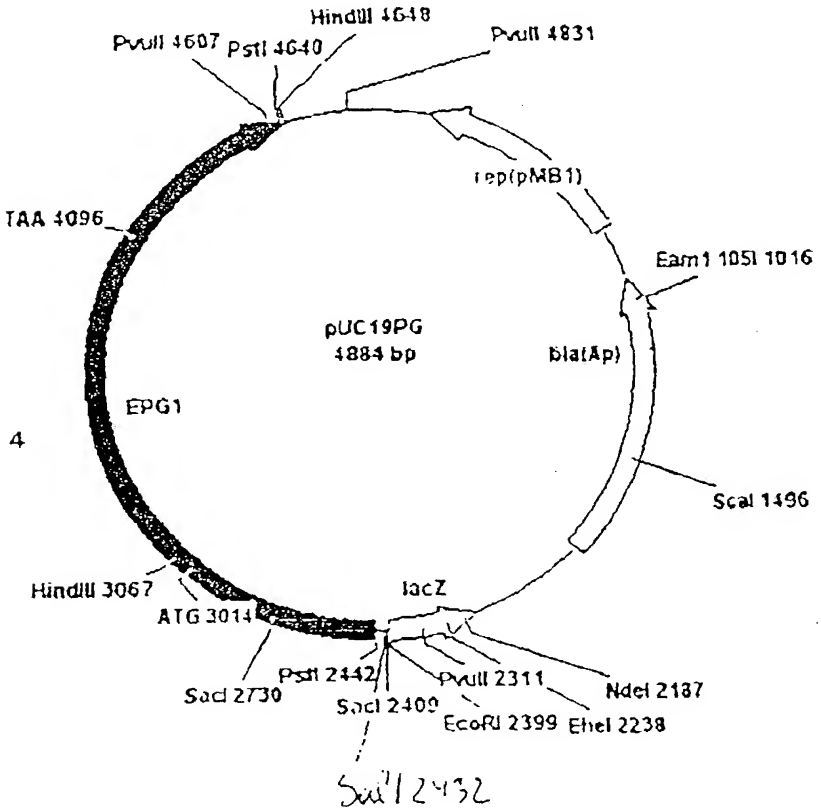

Fig. 4

Plasmid pUC19PG1a:
A 2.735 kb StuI-PvuII DNS restriction fragment from a recombinant LambdaGEM™-12 of the *Kluyveromyces marxianus* gene bank was exchanged in the plasmid pUC19 for the 321 b PvuII fragment.
The bold part corresponds to the DNS fragment from *K.marxianus*. The thin-printed part represents the proportion of the plasmid pUC19.

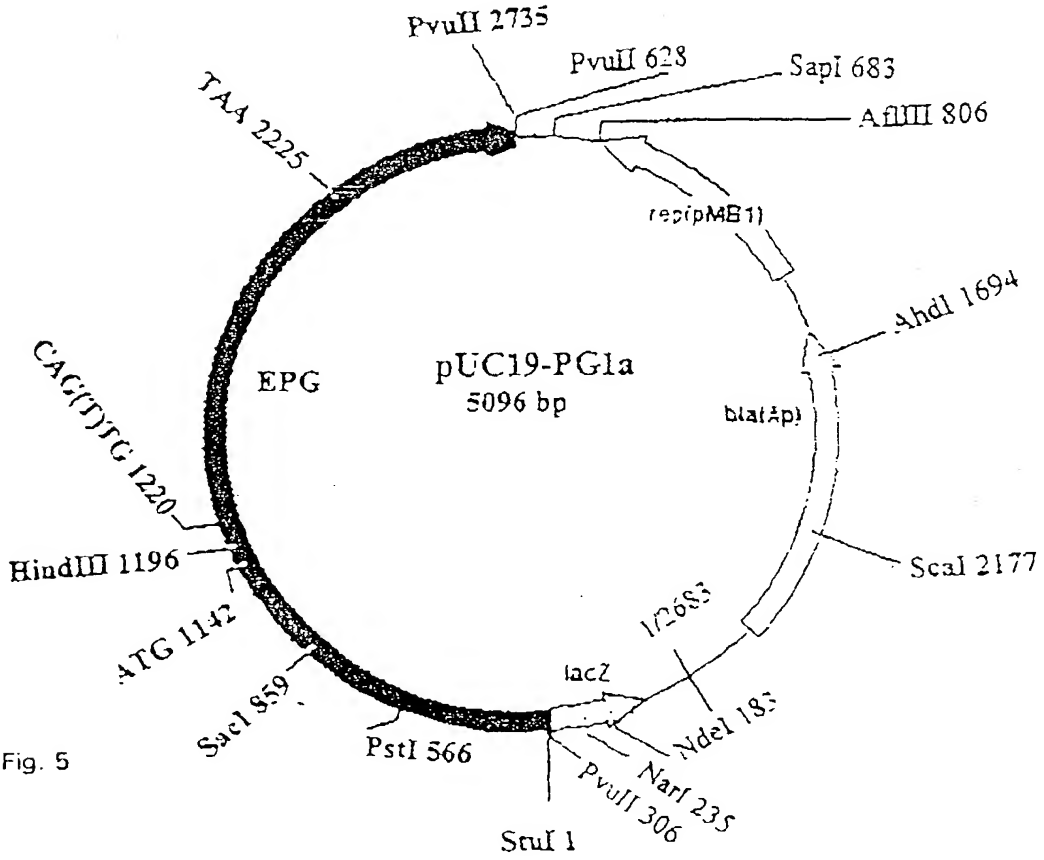

Fig. 5

PCR primers for blunt end cloning of promoter and signal sequence of the EPG1 gene from *Kluyveromyces marxianus*

rPEPG: 5'TTAA**CAGCTG**TCTCTCTTTTCCAATGGAGAAGC 3'
PvuII

vPEPG: 5'TTAAG**AGGCCT**GTCCGATTATAAACTTGCGGC 3'
StuI

Fig. 6

Fig 2: Signal sequence and signal peptid of endoppolygalacturonase from *Kluyveromyces marxianus*

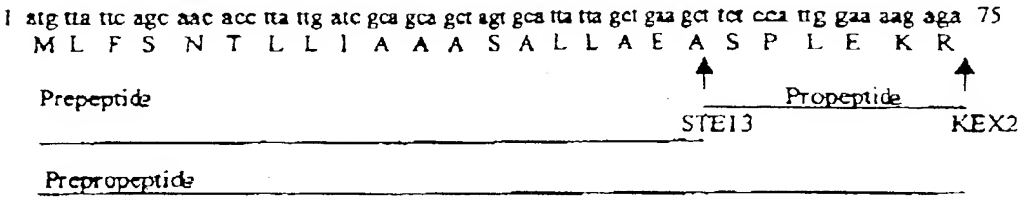

Fig. 7

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

(X) Original () Supplemental () Substitute () PCT

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **"REGULATORY SEQUENCES AND EXPRESSION CASSETTES FOR YEASTS,"** which is described and claimed in the specification

(check one) [ ] which is attached hereto, or
[X] which was filed on March 8, 2002, as United States Application No. _____ and with amendments through (if applicable), or
[ ] in International Application No. PCT/, filed , and as amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information known by me to be material to the patentability of the claims of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code §119 (a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) or §365(b) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATIONS: (ENTER BELOW IF APPLICABLE)			PRIORITY CLAIMED (MARK APPROPRIATE BOX BELOW)	
APP. NUMBER	COUNTRY	DAY/MONTH/YEAR FILED	YES	NO
PCT/EP00/08662	PCT	5 SEPTEMBER 2000	X	
DE 199 43 383.6	Germany	10 SEPTEMBER 1999	X	

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

APPLICATION NUMBER	FILING DATE

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose all information known by me to be material to the patentability of the claims of this application as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS (MARK APPROPRIATE COLUMN BELOW)		
		PATENTED	PENDING	ABANDONED

I hereby appoint the following attorneys and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

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ATTORNEY DOCKET NO. 07038.0004U1

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

(X) Original () Supplemental () Substitute () PCT

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am an original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **"REGULATORY SEQUENCES AND EXPRESSION CASSETTES FOR YEASTS,"** which is described and claimed in the specification

(check one) [ ] which is attached hereto, or
[X] which was filed on March 8, 2002, as United States Application No. _____ and with amendments through (if applicable), or
[ ] in International Application No. PCT/, filed , and as amended on (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information known by me to be material to the patentability of the claims of this application in accordance with Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code §119 (a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) or §365(b) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN APPLICATIONS: (ENTER BELOW IF APPLICABLE)			PRIORITY CLAIMED (MARK APPROPRIATE BOX BELOW)	
APP. NUMBER	COUNTRY	DAY/MONTH/YEAR FILED	YES	NO
PCT/EP00/08662	PCT	5 SEPTEMBER 2000	X	
DE 199 43 383.6	Germany	10 SEPTEMBER 1999	X	

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		PATENTED	PENDING	ABANDONED

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23859
PATENT TRADEMARK OFFICE

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ATTORNEY DOCKET NO. 07038.0004U1

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Inventor's signature: Leopold Döhner Date: 27/05/2002

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Inventor's signature: Sal Date: 31.05.02

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Citizenship: Germany

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1.00

Full name of first inventor: DIETMAR BECHER

Inventor's signature: Dietmar Becher Date: 10.05.2002

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2.00

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Inventor's signature: [signature] Date: 15.05.2002

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Citizenship: Lithuania

3.00

Full name of third inventor: DANGUOLE
~~DANGOULE~~ BARTKEVICIUTE

Inventor's signature: [signature] Date: 21.05.2002

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Same as Residence

Citizenship: Lithuania

ATTORNEY DOCKET NO. 07038.0004U1

4th Full name of fourth inventor: KESTUTIS SASNAUSKAS

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Citizenship: Lithuania

5th Full name of fifth inventor: LEOPOLD DÖHNER

Inventor's signature: Leopold Döhner Date: 27/05/2002

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Post Office Address: Same as Residence

Citizenship: Germany

6th Full name of sixth inventor: SALAH SALIM

Inventor's signature: _____ Date: _____

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Post Office Address: Same as Residence

Citizenship: Germany

SEQUENCE PROTOCOL

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